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APPLICATION NUMBER: *60/413,978*
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PROVISIONAL APPLICATION
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Sir:

Herewith is a PROVISIONAL APPLICATION
Title: METHOD OF TREATING BREAST CANCER

Atty. Dkt. PW 044235/0000002	Unknown
M#	Client Ref

Date: September 25, 2002

including:

1. Specification: 32 pages 1A. ☐ Claim: _____ pages 1B ☐ 1 Abstract pages
2. ☐ Specification in non-English language 3. ☒ Drawings: 6 sheet(s)
4. The invention ☐ was ☒ was not made by, or under a contract with, an agency of the U.S. Government.
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APPLICATION UNDER UNITED STATES PATENT LAWS

Atty. Dkt. No. PW 044235/0000002
(M#)

Invention: METHOD OF TREATING BREAST CANCER

Inventor(s): Matthew J. Naylor, Tiina P. Iismaa, and Christopher J. Ormandy

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SPECIFICATION

Introduction

Postnatal development of the murine mammary gland is systemically controlled by the pituitary-ovarian axis. A small number of hormones control different facets of mammary development, for example, estrogen and growth hormone regulate ductal elongation, progesterone is essential for ductal side branching and alveolar bud formation, while prolactin is critical for lobuloalveolar development and lactogenesis. (Hennighausen and Robinson, 2001). Models of *in vitro* mammary development do not reproduce the extent of mammary development seen *in vivo*, suggesting that other crucial systemic hormones remain to be identified. One candidate is the neuropeptide galanin.

Galanin is a 29 amino acid peptide originally isolated from porcine intestine (Tatemoto et al., 1983) that has been implicated in the control of a number of biological processes including cognition, feeding behaviour, neuroendocrine responses, mitogenesis and nociception (Iismaa and Shine, 1999). Galanin signals through a family of three G protein-coupled receptors, galanin receptors (Galr) 1-3 (Habert-Ortoli et al., 1994; Howard et al., 1997; Wang et al., 1997). The generation of mice carrying a loss-of-function mutation of the galanin gene has enabled investigation into the functions of galanin *in vivo* where it regulates the development of sensory and cholinergic neurons, hippocampal excitibility and modulation of pain response (Holmes et al., 2000, Mazarati et al., 2000, O'Meara et al., 2000, Kerr et al., 2001). Overexpression of galanin produces symptoms characteristic of Alzheimers disease and suppresses epileptic-like induced seizures (Kokaia et al., 2001, Steiner et al., 2001). Galanin knockout mice display reduced prolactin levels during pregnancy that correlated with lactational failure (Wynick

et al., 1998). Overexpression of galanin in the lactotroph induces hyperplasia and consequent hyperprolactinemia (Cai et al., 1999) establishing galanin as a growth factor for the prolactin secreting lactotrophs. Together these data clearly demonstrate an essential role for galanin in the control of neuronal development and function. Galanin also acts as a mitogen for a number of small cell lung cancer cell lines (Sethi and Rozengurt, 1991a, Sethi and Rozengurt, 1991b).

The galanin gene is located at 11q13, and like many genes in this region it is amplified in around 13% of breast tumours (Ormandy et al., 2002). Galanin is expressed by a number of breast cancer cell lines, but expression does not correlate with amplification. In contrast, galanin expression correlates with estrogen and progesterone receptor expression and is regulated by estradiol and progesterone (Ormandy et al., 1998). This observation suggested that galanin's role in mammary gland development may involve more than simple modulation of pituitary prolactin secretion. We have utilised galanin knockout mice, combined with mammary transplantation, whole organ culture and transcript profiling to examine in detail the role of galanin in mammary development. Galanin was found to act directly on the mammary gland via the Jak/Stat pathway to induce epithelial differentiation, and in concert with prolactin to enhance the development of the lobuloalveoli to a much greater extent in organ culture than has previously been achieved. These data establish galanin as a new systemic hormone regulating epithelial cell differentiation during development of the lobuloalveoli.

Results

Galanin and galanin receptors are differentially expressed in the mammary gland

Expression of galanin and Galr 1-3 was examined by RT-PCR using mammary glands collected at various stages of development (Fig. 1). Galanin was expressed at all time points from estrous in virgin mice through to lactation, but significantly was not detected during involution. Galr expression was tightly regulated and coordinated. All three receptors were most highly expressed at day 7 of pregnancy. Galr1 was only detected at this time, Galr2 was also detected at lower levels throughout the later stages of pregnancy and involution, and Galr3 mRNA was also detected during estrous and diestrous. Very low expression of Galr3 could also be detected at 5 days of involution with longer exposure (data not shown).

Prolactin rescues lactational failure in $Gal^{-/-}$ mice, but fails to completely restore lobuloalveloi differentiation

Targeted disruption of the galanin gene results in failure of ductal side branching during puberty and lactation failure following pregnancy (Wynick et al., 1998). Mammary development during pregnancy has not been investigated in $Gal^{-/-}$ mice. At day 12 of pregnancy the amount of alveolar development was decreased in $Gal^{-/-}$ mammary glands compared to galanin wild type ($Gal^{+/+}$) mice (Fig. 2A). This defect continued throughout pregnancy and at the 1st day post-partum, $Gal^{-/-}$ mammary glands showed reduced lobuloalveolar development compared to the normal development observed in $Gal^{+/+}$ mice (Fig. 2B). Histological examination showed that lactation had not commenced in

$Gal^{-/-}$ mice, where small alveoli showed colostrum retention (Fig. 2C). Differentiation of the mammary epithelium was assessed by quantitative analysis of the mRNA levels of several milk protein genes. Early (WDMN-1), mid (β-casein) and late (WAP) stage markers of epithelial cell differentiation were all decreased in $Gal^{-/-}$ mammary glands compared to $Gal^{+/+}$ littermates (Fig. 2E). Examination of the stomach contents of pups showed that 11 of 12 knockout females were unable to lactate following their first pregnancy (Fig. 2D), despite the observation of normal maternal behaviour and suckling of pups. Interestingly this effect was lost following their second pregnancy.

Since homozygous disruption of the galanin gene results in decreased levels of plasma prolactin during pregnancy, we determined whether treatment of $Gal^{-/-}$ mice with prolactin would rescue the defect in lobuloavleolar development and lactation. Treatment with either 0.6 or 1.2 μg of prolactin per 24 hours throughout the duration of pregnancy restored lactation to a point sufficient for pup survival (Fig. 2D). Whole mount investigation demonstrated restoration of lobuloalveolar development comparable to $Gal^{+/+}$ mice (Fig. 2B), but histological examination showed that compared to wild type animals there were many more ducts that had not commenced lactation and which retained colostrum (Fig. 2C). Analysis of milk protein gene expression revealed that prolactin treatment completely failed to rescue the defect in mammary differentiation revealed by the expression of the milk protein genes (Fig. 2F). Thus, although prolactin treatment restored development and lactation to a level sufficient for pup survival, it did not completely restore mammary differentiation to wild type levels.

Galanin does not act via an essential autocrine or paracrine mechanism to regulate mammary development

The incomplete rescue of lobuloalveolar development following prolactin treatment in $Gal^{-/-}$ mice indicated that galanin must act by an additional mechanism to regulate epithelial cell development. The secretion of galanin by the pituitary and coordinated regulation of galanin receptors in the mammary gland during pregnancy suggests a possible endocrine role for galanin, while the expression of galanin in the mammary gland also raised the possibility of an autocrine or paracrine mechanism. To determine whether mammary galanin production is essential for normal development, recombined glands were formed in which the galanin gene was lacking in either the mammary epithelium or stroma, in the context of a normal endocrine background, including circulating galanin levels. Deletion of galanin from the stroma or from the epithelium, or from both, did not recapitulate the failure of lobuloalveolar development seen in $Gal^{-/-}$ mice on the 1st day post-partum (Fig. 3 and data not shown).

Galanin can act directly on the mammary gland to induce alveolar differentiation and proliferation

Next we determined if circulating galanin could act in an endocrine manner via mammary galanin receptors to induce ductal side branching or alveolar proliferation and differentiation. As galanin treatment *in vivo* would indirectly induce mammary development via endocrine regulation of growth hormone, prolactin and progesterone, we utilised an *in vitro* mammary gland explant model of mammogenesis (Plaut et al., 1993).

Ductal side branching similar to that seen during puberty was produced when mammary gland explants were cultured in insulin (I), aldosterone (A) and hydrocortisone (H) (Fig. 4). The addition of 100nM galanin to the medium did not alter ductal or lobuloalveolar development measured by quantitative morphology and histology. When prolactin was added to the culture medium, lobuloalveolar development was observed (Fig. 4), though as noted previously not to the extent observed during pregnancy *in vivo*. The addition of 100nM galanin to IAH-prolactin medium resulted in a 3.8 fold increase in the number of lobuloalveoli per gland (8.6±2.1 IAH+prolactin v. 33.0±6.1 IAH+prolactin+galanin, p=0.005), causing the glands to resemble those observed *in vivo* during pregnancy. Additionally, the size of individual lobuloalveoli in IAH+prolactin+galanin treated glands was also greater than in IAH+prolactin treated glands alone (Fig. 4). These data show that galanin can act directly on the mammary gland to augment prolactin-mediated lobuloalveolar development, establishing galanin as a new endocrine factor active during this phase of development.

Galanin and prolactin induced signalling pathways in the mammary gland

We next investigated activation of the Jak/Stat, MAP kinase and PI3 kinase signalling pathways by prolactin and galanin. As expected, in explants treated with prolactin we saw activation of the Jak/Stat pathway, with an increase in total Stat5 and a dramatic increase in phosphorylated Stat5 in these samples (Fig. 4). Similarly, prolactin treatment activated the MAP kinase pathway, while the level of total ERK1/2 decreased, the levels of phosphorylated ERK dramatically increased in explants receiving prolactin. Examination of the AKT pathway revealed decreased mobility but no increase in total

AKT in explants receiving prolactin, and no increase in phosphorylation of the two residues most important to AKT activation. The decrease in mobility may represent phosphorylation of other sites on the AKT molecule.

Surprisingly, galanin alone was able to activate the Jak/Stat pathway, similar to prolactin (Fig. 4), but in stark contrast to prolactin, galanin did not induce activation of the MAP kinase pathway or alter the mobility of AKT. When explants were treated with galanin and prolactin there were no dramatic changes to the effects produced by either hormone alone. The apparent slight diminution in pERK and increase in AKT and pAKT(T308) in the figure were not consistent between experimental replicates. We examined makers of mammary epithelial cell differentiation by western blot. Again as expected, explants treated with prolactin showed synthesis of the milk proteins WAP and alpha and beta casein. Strikingly, galanin alone produced the greatest induction of milk protein synthesis despite the absence of lobuloalveolar structures (Fig. 4).

Together these results show that galanin, via activation of the Jak/Stat pathway can induce epithelial cell differentiation, measured by milk protein synthesis, in the absence of epithelial cell proliferation, measured by lobuloalveolar development. In contrast prolactin, which activates the Jak/Stat, MAP kinase and possibly PI3 kinase pathways, produces both epithelial cell differentiation and epithelial cell proliferation. Together these hormones allow lobuloalveolar development to proceed *in vitro* to a level beyond that achievable by prolactin alone, and thus further than lobuloalveolar differentiation has previously been taken *in vitro*.

Transcript profiling of galanin and prolactin induced mammary development

We next examined the transcriptional response of the mammary gland to galanin and prolactin using the Affymetrix microarray suite and MGU74Av2 oligonucleotide GeneChips. Using a Venn-Diagram approach genes were placed into sets that showed increased or decreased expression in response to treatment with prolactin (P), galanin (G) or prolactin with galanin (PG) greater then 1.7 fold compared to IAH treatment alone. The assignment of genes to these groups (Fig. 5) was then validated using quantitative RT-PCR. We found that the vast majority of genes fall into one of two groups (Fig. 5A).

The first major set, at the intersection of all three groups, comprises genes that either all decreased or all increased in response to all three treatments. This set represents 136 regulated genes (40% of all regulated genes). Increasing genes in this set include markers of mammary epithelial cell differentiation, such as the milk proteins (WAP, WDMN-1 and 5 casein family members). Other genes in this group include CIS and SOCS2, negative regulators of the Jak/Stat signalling pathway, functional demonstration of activation of the Jak/Stat pathway by galanin. A number of genes with demonstrated roles in mammary development are also regulated by both galanin and prolactin including E74-like factor 5 (Elf5), growth hormone receptor (GHR), insulin-like growth factor 1 (IGF-1), IGF binding protein 5 (IGFBP-5) and helix-loop-helix protein Id2 (Zhou et al. submitted, Tonner et al., 2002, Hadsell and Bonette, 2000, Gallego et al., 2001, Mori et al., 2000).

The second major set contains 154 regulated genes (44%) and is found at the intersection of the prolactin and prolactin with galanin treatment groups. In striking contrast the reciprocal set at the intersection of galanin and prolactin with galanin

treatment groups contains just one gene. Genes in this group include procollagen I alpha 1 & 2, nuclear factor I/X, claudin 5, and zinc finger protein 125.

The remaining groups are small in comparison to those discussed above. A predominantly decreasing group of genes is regulated by galanin, but not by galanin with prolactin. This pattern indicates that the galanin induced change in the expression of these genes is inhibited by prolactin. Six genes are found in the converse group, prolactin induced expression inhibited by galanin. Genes found in these two sets include IGF-binding protein 6 (IGFBP-6), platelet-derived growth factor receptor alpha (PDGFRα), dermatopontin and glucose phosphate isomerase 1.

A very interesting subset of genes were regulated by treatment using prolactin with galanin but not by treatment using either hormone alone, identifying a synergistic effect of these two hormones that is consistent with the synergistic effect of galanin and prolactin on lobuloalveolar development. Genes here include platelet-derived growth factor receptor beta (PDGFRβ), interlukin 1 receptor antagonist and steroidogenic acute regulatory protein.

Taken together these experimental results demonstrate that galanin exerts a previously undiscovered role as a potent mediator of epithelial cell differentiation during lobuloalveloar development through pregnancy.

Discussion

Our results demonstrate that galanin, a neuropeptide critical for neuronal development and function, is also essential for mammary development. Galanin knockout mice display reduced lobuloalveolar development and subsequently fail to lactate. While prolactin treatment restored lactation to a level sufficient for pup survival, lobuloalveolar differentiation remained impaired. Galanin directly induced epithelial cell differentiation in a whole mammary gland explant model and also augmented the prolactin-initiated process of lobuloalveolar development. Galanin activated the Jak/Stat pathway and induced gene expression patterns consistent with cell differentiation, while prolactin activated the Jak/Stat and Map Kinase pathways and induced patterns of gene expression consistent with both differentiation and proliferation. These results support two key findings, firstly that galanin has a previously undiscovered direct endocrine role in mammary development, and secondly that galanin exerts a strong influence on epithelial cell differentiation during lobuloalveolar development. These findings are discussed below.

Galanin's endocrine role is summarised in Figure 6. Galanin exerts both indirect and direct effects on mammary development. The indirect effects stem from galanin's action as a growth factor for the lactotroph- the prolactin producing cells of the pituitary. Via this mechanism galanin controls circulating prolactin levels (Wynick et al., 1998). Prolactin has a well established role in mammary development, where it acts indirectly during puberty to control ductal side branching via regulation of ovarian progesterone (Vomachka et al., 2000), and directly during pregnancy to allow lobuloalveolar development (Brisken et al., 1999).

Galanin's direct effects on the mammary gland arise from its presence in the circulation. Blood serum galanin levels are low in non-pregnant animals and rise during pregnancy to peak at day 12 of pregnancy, from which point they remain elevated until at least day 18 of pregnancy. In the pituitary galanin mRNA levels increase between day 3 to 7 of pregnancy and remain high throughout the rest of pregnancy. Galanin is also produced by the placenta. Decidual galanin expression is more tightly regulated than the pituitary, being detected only from day 7 to 15 of pregnancy and peaking at day 11 (Vrontakis et al., 1992). Comparison of these expression patterns with serum levels show that both the placenta and pituitary contribute to the profile of serum galanin levels throughout pregnancy. Placental production of a variety of hormones is thought to represent a mechanism by which the developing foetus can "hijack" the maternal endocrine system to ensure its nourishment and survival. For example hypophysectomy cannot induce the loss of pregnancy from mid gestation due to support of ovarian steroid hormone production by placental lactogen (Handwerger, 1991). The role of placental hormones can now be extended to the preparation of the mammary gland for lactation via the differentiative influence of placental galanin. Galr mRNA expression in the mammary gland is highest while galanin serum level are rising during the first week of pregnancy, and are then reduced when circulating levels are high, suggesting regulation of receptor levels to control signal flux. Galr2 expression continues throughout pregnancy, but Galr1 and Galr3 expression is lost by mid pregnancy, suggesting Galr2 as the most likely mediator of galanin's effects. Consistent with this null mutation of Galr1 produces no mammary defect (MJN, CJO, A. S. Jacoby and TPI, unpublished data).

Our second key finding is that galanin exerts only a differentiative activity in the mammary gland, which contrasts to prolactin which exerts both proliferative and differentiative action. This conclusion is based upon our analysis of the signalling pathways activated by these hormones and the patterns of gene expression subsequently induced.

Both galanin and prolactin activated the Jak/Stat signalling pathway. Combined loss of Stat5a and Stat5b in the mammary gland results in failure of lobuloalveolar development and the loss of the expression of genes indicative of cell differentiation (Miyoshi et al., 2001). Reduction, but not loss of Jak/Stat activation, as occurs in $PRLR^{+/-}$, $Stat5a^{-/-}$ and $Gal^{-/-}$ animal models results in a similar phenotype of lactational failure at the first pregnancy followed by recovery of lactation following subsequent pregnancies (Liu et al., 1997, Ormandy et al., 1997, Wynick et al., 1998). Stat5 is also activated by the GHR and epidermal growth factor receptor (EGFR) (Gallego et al., 2001), as well as having decreased binding activity in the mammary glands of $Id2^{-/-}$ mice (Mori et al., 2000) and thus integrates the signals from many different pathways. Unlike galanin, prolactin also activated the MAP kinase signalling pathway, seen as a marked increase of ERK phosphorylation. Prolactin also altered the mobility of AKT, though the phosphorylation state of two key sites remained unaltered. The MAP kinase and PI3 kinase signalling pathways have an established role in the regulation of cell proliferation. These pathways coordinate the mitogenic response of almost all growth factor - receptor tyrosine kinase induced signalling, many of which have an established role as regulators of proliferation in the mammary gland. Oncogenes and tumour suppressors in these signalling pathways

include Neu, Ras, EGFR family members, PTEN and AKT (Muller and Neville, 2001, Li et al., 2002).

This contrasting pattern of signal transduction is reflected by the contrasting developmental effects of these hormones. In organ culture galanin alone does not induce lobuloalveolar development but dramatically increases differentiation measured by milk protein gene expression. In contrast prolactin induces lobuloalveolar development and milk protein expression, but not to the extent observed in whole animals. Galanin and prolactin together induce lobuloalveolar development similar in extent to that seen in whole animals and well beyond the extent previously achieved in organ culture. Galanin should be viewed as a hormone essential for full functional differentiation of the mammary gland.

To further examine the effects of galanin on the mammary epithelium we used a Venn diagram analysis of a transcript profiling experiment to examine the response of the explants to hormone stimulation. A striking pattern was observed. The majority of genes regulated by prolactin and/or galanin fell into two major groups.

The first large group showed regulation of expression by all three treatments (prolactin, galanin, prolactin with galanin), indicating that galanin and prolactin acted independently of each other to control their expression. This set contained the milk protein genes, markers of mammary epithelial differentiation and known Jak/Stat target genes. Thus this group appears to represent the independent action of prolactin or galanin via the Jak/Stat pathway. This group also includes GHR, IGF-1 and IGFBP-5. GHR and IGF-1 have well documented roles in the regulation of ductal growth and milk protein expression (Hadsell and Bonette, 2000, Gallego et al., 2001). Galanin may regulate pituitary GH synthesis

and release (Chan et al., 1996) with potential for the regulation of both systemic and local IGF1 production. IGFBP-5 a negative regulator of IGF-1, and controls apoptosis in the mammary gland (Tonner et al., 2002). Another gene in this set is the epithelial specific *ets* transcription factor Elf5. We have recently identified Elf5 as a Prlr regulated gene by transcript profiling of mammary transplants devoid of Prlr (Harris et al. submitted). Further, it has recently been demonstrated that Elf5 is critical for both embryonic and mammary gland development (Zhou et al. submitted) identifying Elf5 as a critical mediator of the transcriptional response to both galanin and prolactin.

The second major group showed regulation of expression when prolactin was included in the treatment, regardless of whether galanin was present. The converse group, genes regulated when galanin was included in the treatment, regardless of prolactin, was almost non-existent. This disparity is probably due to the ability of prolactin to activate signalling pathways, such as the Map kinase pathway, that galanin is unable to activate. Thus this group probably represents prolactin regulated genes via the Map kinase pathway or via the combined signals of the Jak/Stat and Map kinase pathways. The very small size of the converse group indicates that galanin has very little influence via signalling pathways other than those activated in common with prolactin such as the Jak/Stat pathway.

A third interesting group of genes was found which showed regulated expression only when explants were treated with combined galanin and prolactin. These glands showed extensive lobuloalveolar development beyond that produced by prolactin alone, and so this set represents genes regulated either by the synergistic action of these hormones or by the further development of the lobules. Of particular interest is the synergistic

induction of PDGFRβ. While the role of PDGF in normal mammary gland development is unclear, PDGFR mediated signalling can induce the expression of Stat5 and other Stat family members (Valgeirsdottir et al., 1998). PDGF is a potent mitogen for a variety of different cells including some types of mammary derived cells. PDGF also induces IGF-1, as well as the potent mammary oncogene c-myc (Bowman et al., 2001, Carlberg and Larsson, 1996). These data are suggestive of a proliferative role of PDGFRβ in the mammary gland. This finding is particularly interesting because treatment with galanin alone reduces the expression of the closely related PDGFRα, while treatment using combined prolactin and galanin prevents galanin from reducing PDGFRα expression. Antagonism of galanin action by prolactin may be a switch by which cell fate is directed toward proliferation. Conversely, overriding galanin action, which from the serum profiles would occur from mid pregnancy, may force the dimmunition of proliferation and induce cell differentiation. This raises the possibility that galanin may act as a tumour suppressor gene in the mammary gland. Several studies have suggested a similar role for galanin in experimental models of gastric, colon and pancreatic cancer (Iishi et al., 1998, Iishi et al., 1995, Iishi et al., 1994). Further studies are currently aimed at determining if galanin acts as a tumour suppressor in mammary carcinoma.

In summary we have shown that circulating galanin provides a differentiative influence over the mammary epithelium, adding a new member to the small list of systemic hormones that control mammary gland development.

Materials and methods

Animals

$Gal^{-/-}$ mice (Wynick et al., 1998) used in these studies were of the 129OlaHsd genetic background. $Rag1^{-/-}$ mice (Mombaerts et al., 1992) on the inbred C57BL/6J background were purchased from Animal Resource Centre, Perth, Australia. All animals were specific pathogen free and housed with food and water *ad libitum* with a 12 hr day/night cycle at 22°C and 80% relative humidity.

mRNA Isolation

The 4th inguinal mammary gland was frozen in liquid nitrogen before storage at -80°C prior to use. Total RNA was extracted using TRIZOL Reagent (Gibco BRL) according to the manufacturer's instructions.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

First strand cDNA synthesis used avian myeloblastosis transcriptase (Promega) according to the manufacturer's instructions. PCR primers for Galanin (Acc No. NM 010253), Galr1 (Acc No. NM 008082), Galr2 (Acc No. NM 010254), Galr3 (Acc No. NM 015738) and GAPDH (Acc No. M32599) were designed on the basis of mismatch to other genes. The following primers were used in this study: (Galanin) mGal-F1 5'-TGCAGTAAGCGACCATCCAG-3' (forward) and mGal-R1 5'-AGCACAGGACACACGTGCAC-3' (reverse), (Galr1) mGalr1-F1 5'-CGCCTTCATCTGCAAGTTTA-3' (forward) and mGalr1-R1 5'-

CAGGACGGTCTGTGCAGT-3' (reverse), (Galr2) mGalr2-F1 5'-TGCCTTTCCAGGCCACCATC-3' (forward) and mGalr2-R1 5'-GCGTAAGTGGCACGCGTGAG-3' (reverse), (Galr3) Galr3-F1 5'-CCTGGCTCTTTGGGGCTTTCGTG-3' (forward) and Galr3-R1 5'-AGCGCGTAGAGCGCGGCCACTG-3' (reverse), (GAPDH) GAPDH-F1 5'-TGACATCAAGAAGGTGGTGAAGC-3' (forward) and GAPDH-R1 5'-AAGGTGGAAGAGTGGGAGTTGCTG-3' (reverse). The amplification regime consisted of a 94°C 10 min denaturation cycle, followed by 94°C for 25 sec, 58°C for 30 sec, and 72°C for 2 min, for 33 cycles. An elongation step of 72°C for 5 min ended the PCR.

Oligonucleotides for internal hybridisation of PCR products were 5'-AATGGCCACGTAGCGATCCA-3' (Galr1), 5'-GTAGCTGCAGGCTCAGGTTCC-3' (Galr2) and 5'-GTGGCCGTGGTGAGCCTGGCCT-3' (Galr3).

Recombined mammary gland transplantation

Donor mammary tissue (1 mm^3) from $Gal^{+/+}$ or $Gal^{-/-}$ 12 week old mice was inserted into the excised fat pad of $Gal^{+/+}$ or $Gal^{-/-}$ 3 week old mice cleared of endogenous epithelium. This recombined mammary epithelium-stroma complex was then grafted between the abdominal cavity and skin, between the 3rd and 4th mammary glands of 3 week old $Rag1^{-/-}$ mice (Brisken et al., 1998). This procedure resulted in 100% transplant survival with >95% showing ductal outgrowth. Using this method, recombinations of mammary epithelium and stroma were produced that allowed deletion of the galanin gene from stroma and/or epithelium.

Histological analysis

Mammary whole mounts were made by spreading the gland on a glass slide and fixing in 10% formalin solution. Glands were defatted in acetone before carmine alum (0.2% carmine, 0.5% aluminium sulfate) staining overnight. The whole mount was dehydrated using a graded ethanol series followed by xylene treatment for 60 min and storage and photography in methyl salicylate (Bradbury et al., 1995). Morphometric analysis was performed by counting the number of side branches, alveolar buds or lobulo-alveoli per mammary gland (n=5) for explant cultures or from 4 representative fields of view from whole 4th inguinal glands.

Prl treatment of mice

On the morning of the observation of a vaginal plug, 6-8 week old mice were implanted with a 0.25 μl per hour, 28 day mini-osmotic pump (Alzet) containing either unmodified Prl prepared as described (Chen et al., 1998). Either 0.6 or 1.2 μg were delivered per 24 hr. On the first day post-partum maternal behaviour of mothers was observed, pups were examined for the presence of milk and glands were taken for histological analysis.

Mammary explant culture

Four week old BALB/c mice were implanted with estrogen, progesterone and cholesterol pellets (Ginsburg and Vonderhaar, 2000). Following nine days of treatment, the fourth glands were removed and stretched onto siliconized lens paper and placed into petri dishs containing 2 mL of Waymouths 152/1 medium supplemented with penicillin (100 U/ml),

streptomycin (100 μg/ml), gentamycin sulfate (50 μg/ml), 20 mM HEPES, insulin (5 μg/ml), hydrocortisone (100 ng/ml) and aldosterone (100 ng/ml) to monitor ductal side branching, with and without 100 nM rat galanin (Auspep). To assess lobuloalveolar development ovine Prl (Sigma, 1 μg/ml) was added to the medium. Glands were maintained in a tri-gas incubator at 50% O_2 and 5% CO_2 in air. Medium was changed after 24 hr, then every second day for 6 days before morphology and histology were assessed.

Transcript profiling

Total RNA was extracted using TRIZOL Reagent (Gibco BRL), purified using RNeasy Mini Kit (QIAGEN), cDNA synthesis performed using Superscript II (Invitrogen Life Technologies), synthesis of Biotin-labeled cRNA performed using BioArray HighYield RNA Transcript labeling Kit (Enzo Diagnostics) and hybridised to Affymetrix MGU74v2 GeneChips overnight as per manufacturer's instructions. Arrays were performed in duplicate using 4-6 glands per treatment group from two separate replicate experiments. Analysis was performed using the Affymetrix GeneChip v5 software (MAS 5), with treatment groups compared back to IAH treatment as the baseline comparison. Venn diagrams where formed by selecting genes called increasing or decreasing by MAS 5 with a fold change greater then 1.7 compared to IAH. These groups were further restricted by excluding genes with a magnitude fold change of 1.2 induced by the other treatments.

Quantitative RT-PCR

Quantitative PCR was performed using LightCycler technology (Roche). The following primers were used in this study WDMN1, β–Actin, WAP, β-casein, Elf5, GlyCam1, IGF1, Prlr. PCR reactions were performed in 10 μL volume with 1 μL of cDNA, 5 pmoles of each primer and FastStart DNA Master SYBR Green I enzyme mix (Roche) as per manufacturers instructions. Relative quantitation of the product was performed by comparing the crossing points of different samples normalised to an internal control (β-Actin). Each cycle in the linear phase of the reaction corresponds to a two fold difference in transcript levels between samples. Each reaction was performed in triplicate using pooled RNA from the 4-6 mammary glands or the treatment groups utilised for transcript profiling.

Western analysis

Following RNA extraction from mammary glands using TRIZOL Reagent (Gibco BRL), protein was extracted following the manufacturer's instructions. Protein was separated using SDS-PAGE (Bio-Rad Laboratories), transferred to PVDF (Millipore) and blocked overnight with 5% skim milk powder, 2% fetal bovine serum, 50 mM sodium phosphate, 50 mM NaCl and 0.1% Tween 20. Membranes were incubated with one of the following primary antibodies: α-milk protein (Accurate Chemical & Scientific Corporation), α-STAT5a (Upstate Biotech), α-phospo-STAT5, α-phospho-Erk1/2, α-Erk2, α-phospho-AKT (S472), α-phospho-AKT (T308), α-AKT (Cell Signaling Technology) or α-β-Actin (Sigma). 20 μg of protein was loaded per lane except for α-milk protein were 400 ng of protein was loaded. Specific binding was detected using Horseradish peroxidase

conjugated secondary antibodies (Amersham Biosciences) with Chemiluminescence Reagent (PerkinElmer) and Biomax Light Film (Eastman Kodak Company).

Acknowledgments

We thank Dr Ameae Walker for the unmodified prolactin used in the osmotic pumps, and K. Peters, J. Harris, M. Garden-Gardner, D. Lynch, R. Lyons, S. Wittlin and J. Visvader for technical assistance. M.J.N was the recipient of a University of New South Wales, Faculty of Medicine, Dean's Research Scholarship. This work was supported by grants to C.J.O from CDMRP (DAMD 17-99-1-9115), The Cancer Council of NSW and the Australian National Health and Medical Research Council.

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Figure legends

Figure 1.

Galanin and galanin receptor expression in the mammary gland. Expression of galanin and galanin receptors at various developmental stages of mammary gland development by RT-PCR and hybridization of RT-PCR products with an internal oligonucleotide (Galr1-3). Developmental stages are virgin mice at estrous (est.), virgin mice at diestrous (diest.), days 7, 12 and 16 of pregnancy (7D, 12D & 16D pregnant), lactation and 5 days of involution (5D invol.).

Figure 2.

Galanin is essential for mammary gland development. (A-B) Carmine stained whole mounts of 4th mammary glands at (A) day 12 of pregnancy and (B) 1st day post-partum. (C) Haematoxylin and eosin stained 5 μm sections from mammary glands at 1st day post-partum. (D) Examination of milk protein (WDMN-1, β-casein and WAP) mRNA expression by quantitative RT-PCR at the 1st day post-partum. Fold difference in expression levels expressed as $Gal^{-/-}$ verse $Gal^{+/+}$. (E) Examination of milk protein (WDMN-1, β-casein and WAP) mRNA expression $Gal^{-/-}$ treated with prolactin verse $Gal^{+/+}$. (F) Analysis of lactation in $Gal^{+/+}$, $Gal^{-/-}$ and $Gal^{-/-}$ mice treated with prolactin throughout pregnancy. Mammary glands from $Gal^{-/-}$ mice have arrested development compared to wild type littermates. Alveolar proliferation is inhibited at mid pregnancy (A) with a failure of full lobuloalveolar development at the 1st day post-partum (B). Alveoli from $Gal^{-/-}$ mammary glands are less differentiated (C) lactating ducts are clear

and open, while non-lactating less differentiated ducts retain colostrum (contents staining pink with oil drops) and have reduced milk protein gene expression (D). $Gal^{-/-}$ mice treated with prolactin have lactation restored (F) however, lobuloalveoli fail to fully differentiate, indicated by the presence of less differentiated alveoli (C) and failure of milk protein gene expression (E).

Figure 3.

Galanin does not act via autocrine or paracrine mechanisms to regulate mammary gland development. Carmine stained whole mounts of $Gal^{-/-}$ (A,C) & $Gal^{+/+}$ (B,D) epithelium transplanted into the fat pad of $Rag1^{-/-}$ mice cleared of endogenous epithelium. (A,B) virgin (C,D) 1st day post-partum. Inserts haematoxylin and eosin stained 5 μm sections from the same glands. Deletion of galanin gene from the epithelium does not effect normal mammary gland morphology or histology.

Figure 4.

Galanin acts directly on the mammary gland to induce lobuloalveoli development. (W/Mount) whole mounts of mammary glands following whole organ culture *in vitro* after culture in the presence of insulin, aldosterone and hydrocortisone (IAH), with or without galanin and prolactin as indicated. Arrows indicate lobuloalveoli. (H&E) haematoxylin and eosin stained 5 μm sections from the same glands. Western blot analysis of the expression of milk proteins, STAT5, ERK and AKT in mammary glands following IAH, + galanin and/or prolactin treatment. Milk protein (α-casein, β-casein and WAP) expression in explant mammary glands demonstrates that milk protein levels are

increased following galanin and prolactin, prolactin or galanin treatment alone. Increased levels of phosphorylated Stat5 was observed in mammary glands following treatment with galanin and/or prolactin. Galanin alone was not able to induce activation of the MAP kinase pathway. Phosphorylated ERK1/2 was increased in mammary glands treated with prolactin or prolactin + galanin despite a decrease in the total levels of ERK. This demonstrates marked specific activation of MAP kinase signalling in those glands treated with prolactin. Examination of the PI3 kinase pathway revealed decreased mobility but no increase in total AKT in explants receiving prolactin. This decrease in mobility was not due to phosphorylation of the two residues most commonly associated with AKT activation.

Figure 5.

Transcriptional response of the mammary gland to galanin and prolactin. Transcript profiling of whole mammary gland explants. (A) Venn diagram showing the total number of genes found to be increasing or decreasing in response to galanin and prolactin as described in the Materials and Methods. (B) Response of selected genes to IAH + galanin (G), + prolactin (P) or + prolactin and galanin (PG) treatment. IAH + galanin treatment alone is sufficient to induce mammary epithelial cell differentiation as demonstrated by induction of milk protein gene expression.

Figure 6.

Summary of the role of galanin in mammary gland development. The stages of mammary gland development are shown schematically with causative reproductive

events indicated above and descriptions of subsequent morphological changes given above each dashed arrow. Hormone secretion is shown by solid arrows. Regulatory influences on hormones or morphology are indicated by dashed lines that are positive (arrow heads) or negative (lines).

EMBODIMENTS OF THE INVENTION

The invention provides a method for inducing mammary epithelial cell differentiation by administering a therapeutically effective amount of galanin. As such, the method can be used for example, to inhibit mammary tumors or augment milk production in mammals, particularly agricultural mammals such as cows. The invention further provides a method for inhibiting naturally occurring epithelial tumors, such as those where there is no apparent carcinogenic etiologic agent, by administering an inhibitorially effective therapeutic amount of galanin.

Abstract

Null mutation of the galanin gene has shown it to be essential for neuronal development and regulation of pituitary prolactin secretion correlated with failed lactation. Galanin is also expressed by the mammary gland, leading us to investigate the mammary action of galanin in detail. We report that although prolactin supplementation of galanin knockout mice enabled pup survival, lobuloalveolar differentiation remained impaired. In organ culture addition of galanin directly induced epithelial differentiation, but not proliferation, via activation of the Jak/Stat pathway. Supplementation with prolactin and galanin, activated both Jak/Stat and Map kinase pathways and resulted in lobuloalveolar development far beyond that achievable with prolactin alone. Examination of gene expression patterns revealed overlapping, unique and synergistic patterns of transcriptional activation by these hormones. These data establish a new role for galanin as a systemic hormone essential for mammary development during pregnancy.

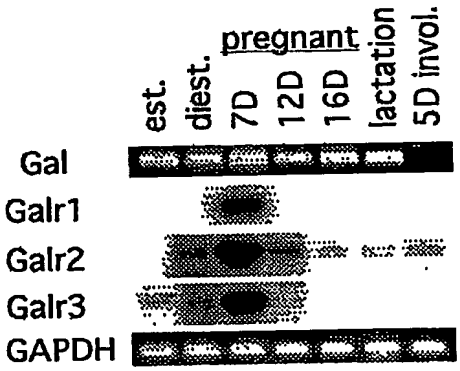

pregnant
est.
diest.
7D
12D
16D
lactation
5D involution
Gal
Galr1
Galr2
Galr3
GAPDH

FIGURE 1

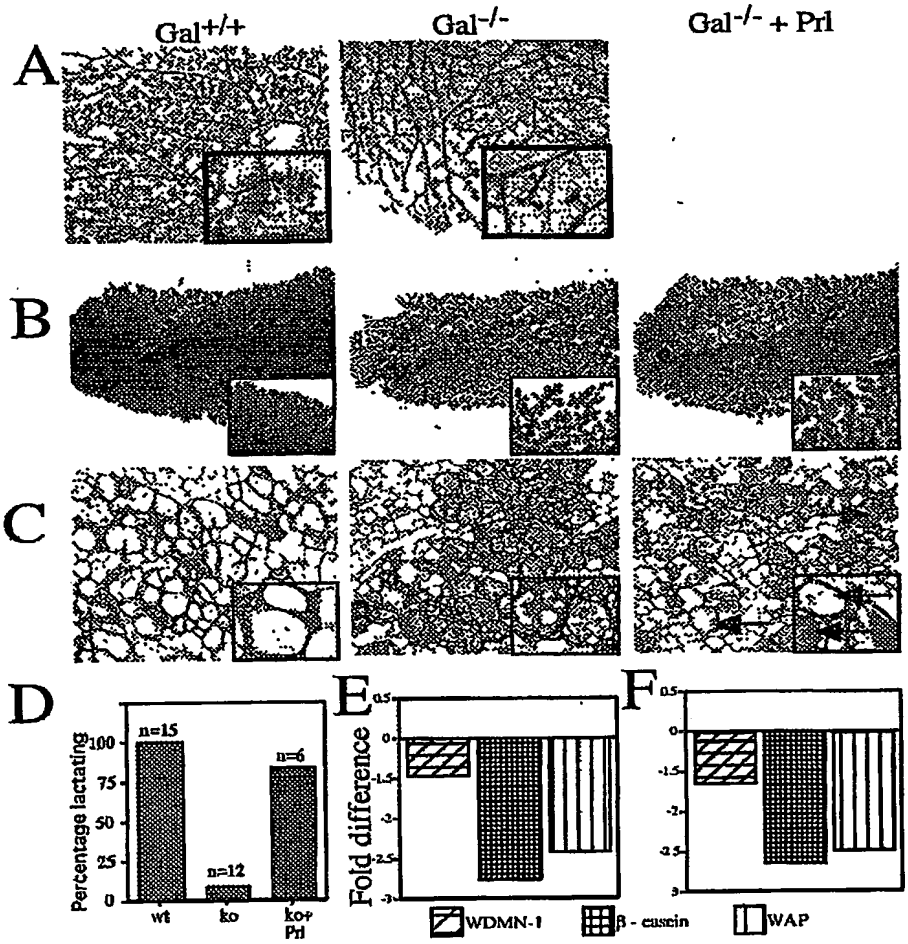

Gal+/+
Gal-/-
Gal-/- + Prl
A
B
C
D
E
F
Percentage lactating
100
75
50
25
0
n=15
n=12
n=6
wt
ko
ko+ Prl
Fold difference
0.5
0
-1.5
-2
-2.5
-3
WDMN-1
β - casein
WAP

FIGURE 2

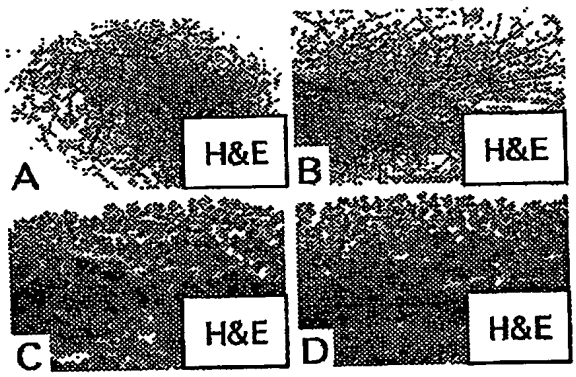


FIGURE 3

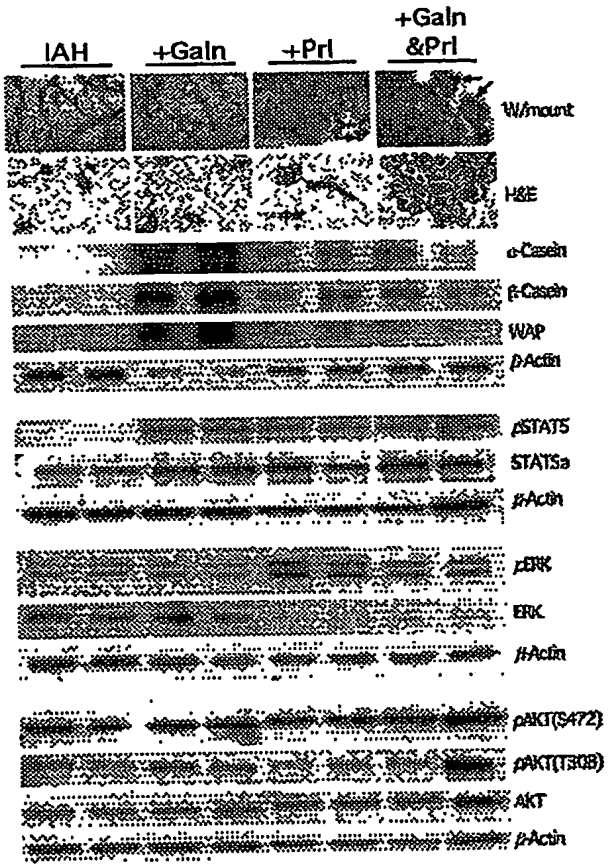
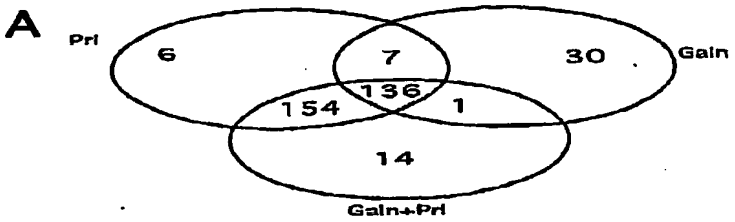


FIGURE 4

A



B

Accession #	Title	Symbol	Annotation	Fold change PG	Fold change G	Fold change P	Q RT-PCR PG	Q RT-PCR G	Q RT-PCR P
PG,G,P									
V00856	whey acidic protein	Wap	milk protein	171	282	251	97	289	126
M93426	glycosylation depend. cell adhes. mol. 1	Glycam1	cell adhesion	88.2	101	133	74	541	257
M36780	casein alpha	Csna	milk protein	73.5	119	81.8			
D10218	casein gamma	Csng	milk protein	58.1	115	89.8			
X04490	casein beta	Csnb	milk protein	41.4	46.8	46.2	100	800	190
M87868	alpha-lactalbumin		milk protein	28.8	77.2	31.1			
M10114	kappa-casein		milk protein	26.4	33.4	30.7			
V00740	casein delta	Csnd	milk protein	14.1	45.9	41.8			
X60367	cellular retinol binding protein I	CRBPI	retinol metabolism	7.41	5.7	7.11			
AF049702	E74-like factor 5	Elf5	transcription factor	6.19	7.11	7.26	7.1	29.4	9.1
X93037	WDMN-1	Expi	milk protein	3.78	10.8	7.41	4	18	17
X04480	insulin like growth factor 1	IGF1	growth factor	2.34	1.69	2.69	1.51	1.89	2.1
M31660	growth hormone receptor	Ghr	GH signaling	3.66	1.51	2.87	6.01	3.01	4.26
L12447	IGF binding protein 5	Igfbp5	IGF signaling	3.07	3.66	3.51			
M98337	milk fat globule-EGF factor 8	Mfge8	cell adhesion	2.81	4.99	3.94			
X95279	spot14	SP14	lipogenesis	2.59	1.94	2.67	1.2	1.32	3.4
M81445	connexin 26	Cx26	gap junction	2.36	2.04	2.35			
AF077861	helix-loop-helix protein Id2	Id2	transcription factor	1.79	1.94	1.89			
X16450	plasminogen activator inhibitor 2	PAI-2	protease inhibitor	-5.6	-8.5	-4.4			
M32660	plasminogen activator inhibitor 1	PAI-1	protease inhibitor	-3.1	-1.6	-2.6			
D10837	rig receptor gene	rig	tumor-suppressor	-2.5	-1.5	-1.9			
PG,P not G									
M19681	PDGF-inducible protein (JE) gene	JE	chemoattractant	2.98	-1.5	2.69			
U03419	procollagen, type I, alpha 1	Col1a1	cell adhesion	2.33	-1.2	2.31			
D29678	cyclin-dependent kinase 5	Cdk5	cell cycle	1.43	1.16	2.23			
X58251	procollagen, type I, alpha 2	Col1a2	cell adhesion	2.19	-1.4	2.16			
L42115	solute carrier family 1, member 7	Slc1a7	transport	2.01	-1.1	2			
Y07868	nuclear factor I/X	Nfix	transcription factor	1.73	1.13	1.87			
U82758	claudin 5	Cldn5	tight junction	2.08	-1	1.85			
AI154711	zinc finger protein 125	Zfp125	DNA binding	-2.2	1.1	-8.1			
D50111	myocyte enhancer factor 2B	Mef2b	transcription reg.	-2.5	-1	-2			
G not P not PG									
AV295044	glucose phosphate isomerase 1 complex	Gpi1	glycolysis	-1.5	1.72	-1.8			
AA178826	dermatopontin	Dpt	cell adhesion	1.29	-2.6	1.92			
X81584	IGF binding protein 6	Igfbp6	IGF signaling	1.79	-2.3	1.23			
M57684	PDGF receptor alpha	Pdgfra	Tyr kinase receptor	1.08	-1.2	1.2			
P not PG not G									
L19998	beta Ig-h3		cell adhesion inhib.	-1.1	-1.3	1.81			
J03877	granzyme G	Gzmg	proteolysis	-1.2	1.02	-2.5			
PG not P not G									
X04967	PDGF receptor beta	Pdgfrb	Tyr kinase receptor	1.96	-1.5	-1.2			
L32838	interleukin 1 receptor antagonist	IL-1ra	IL81 signaling	1.99	-2.5	1.17			
D38046	topoisomerase (DNA) II beta	Top2b	DNA metabolism	1.84	1.05	-1.2			
AB03034	ryanodine receptor 3	Ryr3	calcium homeostasis	-2.1	-1.1	1.01			
AV302016	steroidogenic acute regulatory protein	Star	steroid hormone biosyn	-1.9	1.02	-1			

FIGURE 5

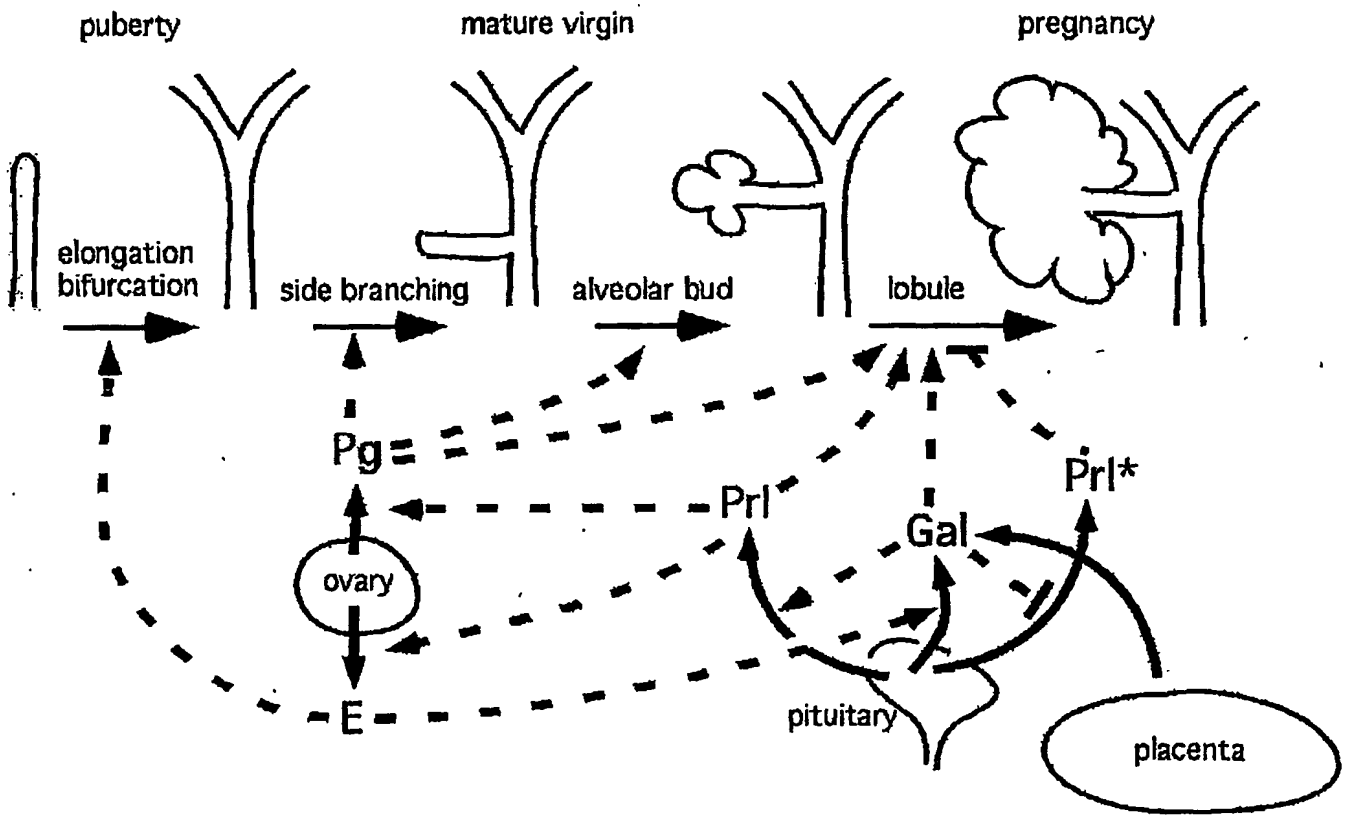

puberty
mature virgin
pregnancy
elongation
bifurcation
side branching
alveolar bud
lobule
Pg
Prl
Prl*
Gal
ovary
E
pituitary
placenta

FIGURE 6